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Expression and significance of p53 and mdm2 in patients with leukoplakia cancer

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ABSTRACT

Objective: To study the relationship of the expressions of p53 and mdm2 in leukoplakia cancer.**Methods:** RT–PCR was used to detect the mRNA of p53, mdm2 in patients with leukoplakia cancer. The frequencies of p53, mdm2 in peripheral blood were detected by flow cytometric analysis. **Results:** The expression of p53mRNA in normal oral mucosa, simple oral leukoplakia, no–simple oral leukoplakia and leukoplakia cancer were 7.7%, 27.3%, 33.3%, 56.8%, respectively. The frequencies of p53 in normal oral mucosa, simple oral leukoplakia, no–simple oral leukoplakia and leukoplakia cancer were $(0.3 \pm 0.1)\%$, $(1.6 \pm 0.9)\%$, $(1.9 \pm 1.1)\%$, $(3.4 \pm 1.8)\%$. The expression of mdm2 mRNA in normal oral mucosa, simple oral leukoplakia, no–simple oral leukoplakia and leukoplakia cancer were 0.0%, 6.8%, 11.1%, 37.8%, respectively. The frequencies of mdm2 in normal oral mucosa, simple oral leukoplakia, no–simple oral leukoplakia and leukoplakia cancer were $(0.1 \pm 0.1)\%$, $(0.8 \pm 0.6)\%$, $(1.2 \pm 0.8)\%$, $(1.2 \pm 0.8)\%$. There was a positively correlation between p53 mRNA and mdm2 mRNA. **Conclusions:** The positive rate of p53 and mdm2 cells in the peripheral blood increases in patients with leukoplakia cancer tissue and has positive correlation with the severity of leukoplakia cancer.

1. Introduction

The mutant p53 gene and mdm2 gene are found in the majority of human tumor tissues, and the relationship between p53 and mdm2 has become the focus of studies. Few studies focuses on p53 and mdm2 in the oral mucosa leukoplakia. In this study RT–PCR and flow cytometric analysis were used to detect the mRNA of p53, mdm2 in patients with normal oral mucosa, simple oral leukoplakia, no–simple oral leukoplakia and leukoplakia cancer, to explore their role in the carcinogenesis of oral leukoplakia.

2. Materials and methods

2.1. Clinical data

A total of 110 oral leukoplakia patients diagnosed by clinical and pathological examination were selected. There were 69 males and 41 females, aged from 23 to 83 years, with disease course from 2 months to 10 years. Standard of clinical manifestations and classification were according to the criteria by WHO in 1996, pathological classification was according to Pindborg criteria^[1].

2.2. Reagents

Trizol (Shanghai Sangon Biological Engineering Co., Ltd.), fluorescein isothiocyanate, lymphocyte separation medium (Langton Biotechnology Co., Ltd.), mouse anti–human p53 monoclonal antibody, mouse anti–human

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mdm2 monoclonal antibodies (Shanghai Sangon Biological Engineering Co., Ltd.), RT-PCR reagent kit (Wuhan Boster Biological Engineering Co., Ltd.), PCR thermal cycler (Bio-Rad, USA), flow cytometry (Bio-Rad, USA).

2.3. Specimen collection

Two days after admission, early morning fasting blood samples were obtained from the forearm vein of the patients then were placed in the heparinized tube.

2.4. RT-PCR detection of p53, mdm2 expression

Specimens were removed from the -80°C refrigerator, and the total RNA was extracted by the instructions of Trizol reagent kit, to determine the quality and concentration of the extracted RNA. The volume of $2\ \mu\text{g}$ RNA was calculated according to the concentration. Reverse transcription was conducted in accordance with kit. The primers were as follows:

p53 primers: Upstream 5'-CTAACC GCGGT CCTTCC CAGAAA CCTAC-3', downstream 5'-TACAGTCAGAGCCAACCTCAGGCG-3', a total of 409 bp.

mdm2 primers: upstream 5'-AATCATCGGACTCAGGTA-3', downstream 5'-CTTCACTAAGGCTATAATCTTC-3', a total of 450 bp.

β -actin as the internal reference: upstream 5'-CAAGGCCAACC GCGAGAAGATG-3', downstream 5'-GTCCAGGGCGACGTAGCACAGC-3', amplification length was 330 bp.

They were amplified in the PCR instrument. After 1.5% agarose gel electrophoresis, the photo were observed with the DC2000 gel imaging analyzer. Compared with Marker, if there were positive bands at 409 bp, 450 bp and 330 bp, it was determined as p53, mdm2 and β -actin mRNA positive expression respectively; If there was no positive band appears in the corresponding area, it was defined as negative. And the optical density value of the bands was analyzed by Quantity One software. The β -actin optical density values were used as internal reference for normalization of the absorbance value of p53 and mdm2 mRNA. The relative content of p53 and mdm2 mRNA expression were obtained.

2.5. Proportion of p53, mdm2 cells in peripheral blood

PBS was diluted by an equal volume, peripheral blood mononuclear cells was obtained from human lymphocyte separation medium. Cell number was adjusted to $1 \times 10^6/\text{mL}$, washed with PBS twice. After centrifuged at 1 000 rpm the supernatant was removed. 1:100 diluted mouse anti-human mdm2, p53 monoclonal antibody $100\ \mu\text{L}$ were added. They were bathed at room temperature for 30 min. After washed

with PBS 2 times the supernatant was discarded, 1:20 dilution of mouse monoclonal antibody FICT-IgG $100\ \mu\text{L}$ marked mdm2 and p53 were added. They were bathed at 37°C for 30 min, then the supernatant was discarded, washed in PBS twice. One mL PBS liquid were added, and analyzed after 400 mesh screen filter. The proportion of p53, mdm2 cells was detected by flow cytometry.

2.6. Statistical analysis

All data were analyzed by SPSS13.0 statistics software. And the data were expressed as mean \pm SD values. *t*-test was applied in the comparison between two groups. Correlation analysis was analyzed with the Speraman correlation analysis. Kaplan-Meier method was used for prognosis analysis, $P < 0.05$ was considered as statistical significance.

3. Results

3.1. RT-PCR detection of p53, mdm2 mRNA expression in different tissues

Among 13 cases of normal oral mucosa, there was 1 case with p53 mRNA positive expression, with the positive rate as 7.7%; Among the simple oral leukoplakia, there were 12 cases with p53 mRNA expression, with the positive rate as 27.3%; Among theno-simple oral leukoplakia, there were 15 cases with p53 mRNA expression, with positive rate as 33.3%; Among the leukoplakia cancer, there were 21 cases with p53 mRNA expression, with the positive rate as 56.8%. The expression of mdm2 mRNA in normal oral mucosa, simple oral leukoplakia, no-simple oral leukoplakia and leukoplakia cancer were 0.0%, 6.8%, 11.1%, 37.8%, respectively. The differences in expression of p53, mdm2 mRNA were statistically significant among the simple oral leukoplakia group, no-simple oral leukoplakia and leukoplakia cancer group ($P < 0.05$). The differences in expression of p53, mdm2 mRNA were not statistically significant between simple oral leukoplakia group and the no-simple oral leukoplakia group ($P > 0.05$) (Figure 1).

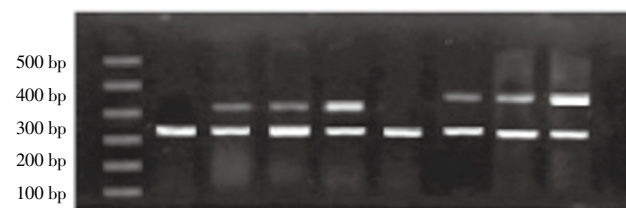


Figure 1. RT-PCR result.

p53: 409 bp, mdm2: 450 bp, β -actin: 330 bp.

3.2. Proportion of p53, mdm2 in peripheral blood in each group were detected by flow cytometric analysis

The frequencies of p53 in normal oral mucosa, simple oral leukoplakia, no-simple oral leukoplakia and leukoplakia cancer were $(0.3 \pm 0.1)\%$, $(1.6 \pm 0.9)\%$, $(1.9 \pm 1.1)\%$, $(3.4 \pm 1.8)\%$, respectively. The frequencies of mdm2 in normal oral mucosa, simple oral leukoplakia, no-simple oral leukoplakia and leukoplakia cancer were $(0.1 \pm 0.1)\%$, $(0.8 \pm 0.6)\%$, $(1.2 \pm 0.8)\%$, $(1.2 \pm 0.8)\%$. The expression of p53 and mdm2 cells in the leukoplakia cancer group was significantly higher than in the normal group, simple leukoplakia and abnormal white group ($P < 0.05$).

3.3. Relationship of p53, mdm2 and clinical pathological features of patients with leukoplakia cancer

For 37 patients with leukoplakia, p53 had a positive correlation with lymph node metastasis and pathological grading process, but had nothing to do with the skin lesions, clinical type and pathological grade (Table 1).

Table 1

Relationship between P53, mdm2 and clinical pathological features of leukoplakia.

Groups	n	p53/ β -actin [M (P5–P95)]	mdm2/ β -actin [M (P5–P95)]
Position			
Cheek	8	0.623(0.525–0.678)	0.463(0.325–0.498)
Ventral tongue	26	0.667(0.425–0.776)	0.478(0.345–0.567)
Other	3	0.594(0.486–0.588)	0.449(0.378–0.546)
Clinical classification			
Homogeneous	7	0.612(0.558–0.738)	0.427(0.364–0.512)
Verrucous	11	0.642(0.525–0.766)	0.442(0.421–0.567)
Particle	9	0.607(0.496–0.776)	0.473(0.410–0.469)
Ulcer	10	0.614(0.425–0.733)	0.474(0.325–0.535)
Lymph node metastasis			
without	17	0.546(0.425–0.688)	0.352(0.325–0.414)
with	20	0.743(0.578–0.884)*	0.524(0.480–0.567)*
Pathological grade			
OSCC I	12	0.570(0.476–0.768)	0.397(0.336–0.426)
OSCC II	13	0.584(0.425–0.776)	0.434(0.325–0.460)
OSCC III	12	0.677(0.457–0.884)*	0.447(0.379–0.567)

* Compared with the same group, $P < 0.05$.

3.4. Correlation analysis of p53 and mdm2 expression

A total of 21 cases had p53 mRNA positive expression, and 12 had mdm2 mRNA expression. Among them, 12 cases had both p53 mRNA and mdm2 mRNA expression. Spearman rank correlation analysis showed positive correlation between p53 mRNA and mdm2 mRNA ($r = 0.636$, $P = 0.006$).

3.5. Survival analysis of p53 and mdm2 expression

In 37 cases with leukoplakia cancer, if the p53 expression was positive, the median survival time was 8 months; if it was negative, the median survival time was 13.5 months, and

the difference was significant statistically. Kaplan–Meier survival analysis curve revealed poor prognosis (Figure 2). If the mdm2 expression was positive, the median survival time was 7.5 months, if it was negative, the median survival time was 12 months, and the difference was significant statistically. And Kaplan–Meier survival analysis curve also suggested poor prognosis (Figure 3).

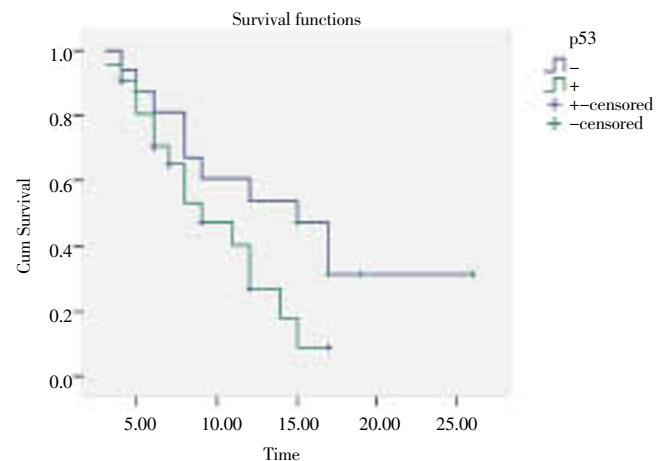


Figure 2. Survival curve analysis of p53 mRNA.

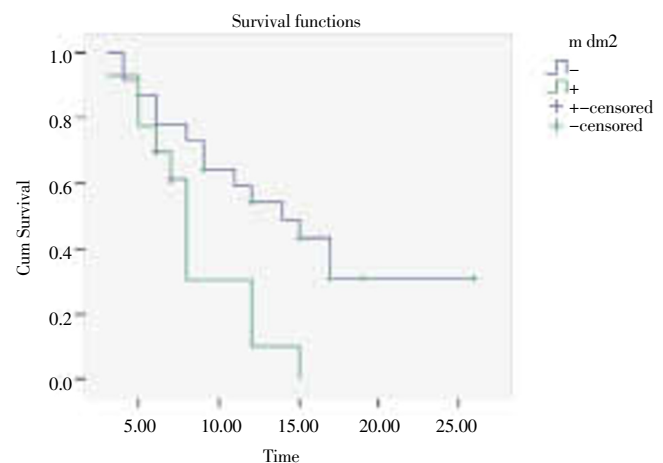


Figure 3. Survival curve analysis of mdm2 mRNA.

4. Discussion

White spot (leukoplakia) refers to the white or off white keratosis lesions of plaque damage to the mucosal epithelium. This pathological plaque can not be erased, and there is a potential of the malignant transformation.

Oral leukoplakia is a precancerous lesions in the oral mucosal epithelial. Dong *et al*[2] reported 17%–35% oral epithelial squamous cell carcinoma is the development of oral leukoplakia. So the treatment and prognosis of oral leukoplakia has great significance for the prevention of oral cancer. At present, the treatment of leukoplakia is still in the exploratory stage, so long-term follow-up is an important mean to monitoring the cancer patients, and it play a role in the prevention and treatment of malignant transformation of oral leukoplakia. The studies confirmed[3,4] that it may be the

maladjustment of epithelial network which made of a variety of cells and cytokines resulting in a white spot disease and thus carcinogenesis.

p53 is a tumor suppressor gene which can induce cell growth arrest and the apoptosis differentiation and DNA repair. Wild-type p53 can inhibit cell division and proliferation, prevent cell malignant transformation. Mutant p53 can lead to tumorigenesis and invasion *in vivo*, prompting cells to produce anti-apoptotic and the capacity of anti-chemotherapy and anti-radiotherapy. Kovesi consider that the different expression of p53 in oral leukoplakia showed the increased genomic instability is consistent with oral leukoplakia cancer clinical manifestations. The mdm2 has a highly tumorigenicity and its amplification and expression can be found in a variety of tumors^[5,6]. The main function of the mdm2 protein is direct bonding to acidic activation domain of the p53 protein and then forming the p53–mdm2 complex, thereby inhibiting wtp53 mediated transcriptional activation function. In this study, we observed the changes of serum p53 and mdm2 expression levels in different types of patients with oral leukoplakia to explore its clinical significance.

The result showed the expression of p53mRNA in normal oral mucosa, simple oral leukoplakia, no-simple oral leukoplakia and leukoplakia cancer were 7.7%, 27.3%, 33.3%, 56.8%, respectively. The frequencies of p53 in normal oral mucosa, simple oral leukoplakia, no-simple oral leukoplakia and leukoplakia cancer were $(0.3 \pm 0.1)\%$, $(1.6 \pm 0.9)\%$, $(1.9 \pm 1.1)\%$, $(3.4 \pm 1.8)\%$. The p53 positive expression rate were in an ascending order from normal oral mucosa, simple oral leukoplakia, no-simple oral leukoplakia to leukoplakia cancer. In patients with leukoplakia cancer, the p53 expression rate and intensity was higher in the pathological high-grade group than in the low-grade group. The p53 positive group was with significantly higher lymph node metastasis rate than the negative group, which suggested that abnormal p53 gene had a correlation with the occurrence of oral leukoplakia cancer, the differentiation of cancer cells and the malignant degree. Liu^[7] also confirmed that p53 gene mutation result in the overexpression of p53 protein which has a correlation with the occurrence and development of oral precancerous lesions. The mdm2 positive expression in leukoplakia cancer were significantly higher than in normal oral mucosa, simple oral leukoplakia, no-simple oral leukoplakia group, and the percentage of cells expression were in an ascending order with the lesions; The mdm2 expression in the lymph node metastasis was significantly higher than that without lymph node metastasis, which showed the mdm2 gene overexpression is more obvious in tumor metastasis. Jiang *et al*^[8], Li also reported mdm2 tumor play an important role in the occurrence and development of tumors, there was a correlation between them. The Spearman rank analysis showed the p53 and mdm2 mRNA were positively correlated in patients with leukoplakia. P53 and mdm2 may participate in the process of the oral leukoplakia cancer and then induce the occurrence of oral leukoplakia cancer.

There are numerous studies which confirmed the correlation between mutant p53 and mdm2. The mutant p53

can not repair damaged DNA, at the same time the mdm2 was activated and then inhibit the expression of the tumor suppressor gene p53, which leading to repeated cycles of mutant p53 and mdm2 chronic accumulation, and ultimately cause malignant transformation and proliferation of cells^[9,10]. Wang^[11] confirmed that p53, mdm2 protein expression are closely related to the occurrence and development of oral lichen planus and oral squamous cell carcinoma. After the follow-up, survival analysis showed that high expression of p53 and mdm2 has poor prognosis in patients with leukoplakia, which suggested that p53 and mdm2 can be the indicators of the prognosis and predicting the outcome of oral leukoplakia cancer.

In summary, the occurrence, development and prognosis of oral leukoplakia cancer may have a correlation with p53 and mdm2 gene expression. So P53 and mdm2 can be a biomarkers for the occurrence and development and prognosis assessment of leukoplakia cancer, which has a great significance for guiding the leukoplakia cancer clinical treatment and improve the prognosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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